

Original  
articleThe vagina has reducing environment sufficient for activation of *Trichomonas vaginalis* cysteine proteinases

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**Background:** *Trichomonas vaginalis*, a worldwide distributed sexually transmitted protozoan, is remarkable for synthesis of numerous, distinct cysteine proteinases, the significance of which is evidenced by the presence in vivo of soluble proteinases in secretions and antiproteinase antibody in serum of patients with trichomonosis. These proteinases purportedly play a role in host parasitism and immune evasion.

**Objective:** It is known that for cysteine proteinases to be functional, they must be activated by disulphide reducing reagents. Whether or not the host vaginal environment has the reducing environment essential for activation of the trichomonad cysteine proteinases is unknown. Our goal, therefore, was to determine whether or not vaginal secretions had sufficient reducing power to activate the trichomonad proteinases.

**Methods:** 48 vaginal washes (VWs) from patients were assayed for reducing equivalents and a score in dithiothreitol (DTT) reducing equivalents was assigned to each VW. Activation of trichomonad cysteine proteinases was then tested under the range of reducing equivalents detected from VWs. The possible protective effect of hydrogen peroxide, an oxidising agent produced by some *Lactobacillus* species, on proteinase activity was also determined.

**Results:** Nine of 48 VWs (18.7%) possessed  $\leq 10 \mu\text{M}$  DTT reducing equivalents, four VWs (8.3%) had from 20  $\mu\text{M}$  DTT to 40  $\mu\text{M}$  DTT reducing equivalents, and most (50%) were between 10  $\mu\text{M}$  to 15  $\mu\text{M}$ . Overall, the range in VWs was from  $\sim 10 \mu\text{M}$  to 40  $\mu\text{M}$  reducing equivalents. Importantly, data suggest differential proteinase activation over this in vivo range of reducing level. Only two *T vaginalis* cysteine proteinase activities were stimulated at 2.5  $\mu\text{M}$  DTT in contrast with all proteinase activities present at 40  $\mu\text{M}$  DTT, albeit quantitatively diminished compared with the activity at 1 mM DTT, the concentration routinely used in vitro. Finally, hydrogen peroxide reversibly neutralised all trichomonad proteinases.

**Conclusions:** These results show that the vagina of women has a reducing environment adequate for activation of trichomonad proteinases. The data underscore that the host environment plays a role in the host-parasite interrelation. Finally, hypotheses can now be formulated to help explain resistance and susceptibility to infection commonly reported among women and between men and women with trichomonosis.

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Keywords: *Trichomonas vaginalis*; vagina; cysteine proteinase

## Introduction

The host-*Trichomonas vaginalis* interrelation is exceedingly complex. Infection with this sexually transmitted parasite often leads to unpredictable outcomes in symptomatology<sup>1-3</sup> that range from a carrier state to severe foul smelling discharge, irritation, and discomfort associated with inflammation and tissue cytopathology.<sup>1-3</sup> It is now appreciated that patients with trichomonosis (vaginitis) are at higher risk for HIV seroconversion,<sup>4,5</sup> for adverse outcomes during pregnancy,<sup>6-8</sup> and, possibly, for cervical cancer.<sup>9,10</sup> The identity of factors, either of host or parasite origin, responsible for the variations in host responses to *T vaginalis* infection remains unknown.

Recently, trichomonad cysteine proteinases have been recognised as virulence factors.<sup>11-16</sup> The parasites generate numerous cysteine proteinases,<sup>17-19</sup> many of which are secreted during normal cultivation in complex medium. The in vivo relevance of the proteinases is affirmed by the detection of proteinases and

antiproteinase antibody in vaginal secretions and in sera of women with trichomonosis, respectively, but not women who are uninfected or who have other sexually transmitted diseases.<sup>11,16,20</sup> These enzymes probably contribute to numerous virulence properties of *T vaginalis* organisms, such as acquisition of nutrients,<sup>21</sup> subversion of host immune responses,<sup>13,16</sup> and, through unknown mechanisms, parasite cytoadherence.<sup>12</sup>

Substrate degradation by cysteine proteinases requires breakage of disulphide bonds, as occurs under reducing conditions.<sup>17-19,22</sup> In this study, we have attempted to address the question of whether the vaginal environment of patients has sufficient reducing levels to activate trichomonad cysteine proteinases. This is important because all studies performed to date have activated the cysteine proteinases by addition of reducing reagents such as DTT. Equally important, it has been hypothesised that the host environment may play a role in regulating, both quali-

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tatively and quantitatively, the numerous trichomonad proteinases thereby contributing to a chronic, non-self limiting infection and minimal manifestation of tissue cytopathology.

In this report we show that vaginal secretions obtained from patients with trichomonosis do indeed possess an environment adequate for activation of the cysteine proteinases. It is noteworthy that the reducing levels produced differential activation or quantitatively distinct amounts of proteinases. These data reinforce the idea that variations in reducing levels in vaginal secretions play a role in infection by *T vaginalis* and possibly in disease outcome. The observations made in this report point to future questions that must be answered to fully understand this complex host-parasite interaction and to meaningfully develop future infection and/or disease intervention strategies.

## Materials and methods

### CULTURES

*T vaginalis* isolates IR78, NYH 286, T048, and T068-II were grown at 37°C in trypticase yeast extract maltose (TYM) supplemented with 10% heat inactivated horse serum (HIHS), as described before.<sup>23 24</sup> To optimally visualise for trichomonad cysteine proteinase activities in substrate gels, as described below, isolate T068-II was also grown under low iron conditions in the presence of 30 µM 2,2-dipyridyl (2,2-DP).<sup>25 26</sup> Organisms ( $2 \times 10^7$ ) were collected at mid to late logarithmic phase of growth<sup>23</sup> by centrifugation at  $500 \times g$  for 5 minutes after washing three times in ice cold phosphate buffered saline (PBS). Cells were either used immediately or frozen at -70°C until needed.<sup>16</sup> Identical proteinase patterns were obtained with lysates derived from either fresh or frozen parasites, as before.<sup>16</sup>

### DETERMINATION OF REDUCING LEVEL IN HUMAN VAGINAL WASHES

To maximise detection of reducing ability, vaginal washes (VWs) were obtained from the source previously reported by us and processed as described before.<sup>11 27</sup> Briefly, a volume of 5 ml of a VW was obtained in sterile PBS by rinsing the vaginal wall numerous times. To this VW a large swab, which was utilised to scrape the vaginal wall, was then immersed in the VW from the same patient. VW was then clarified as previously described by centrifugation at  $500 \times g$  to remove large cellular debris followed by centrifugation at  $17\,500 \times g$  to remove additional insoluble debris. The VWs were kept stored at -70°C until utilised. Upon thawing and just before use, VWs were filter sterilised through 0.22 µm acrodisc filters (Gelman Sciences, Ann Arbor, MI, USA).

The reducing potential of patient VWs was determined spectrophotometrically at 412 nm by comparing readings with a standard curve derived from a buffer comprised of known concentrations of DTT in Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid; DTNB))<sup>28</sup> (100 mM TRIS-HCl, pH 8.0, 100 mM

EDTA, 10 mM DTNB, 50 mM NaPO<sub>4</sub>, pH 7.0, 10% sodium dodecylsulphate (SDS)). Reducing values of VWs diluted in DTNB were obtained from the average of triplicate determinations, and values were expressed as µM DTT equivalents. Reducing equivalents  $\leq 10$  µM DTT were below the detection limit of DTNB and, therefore, represented as  $\leq 10$ .

### DETECTION OF TRICHOMONAD CYSTEINE PROTEINASE ACTIVITY

Protocols for preparation of parasite lysates and for substrate gel electrophoresis have been extensively described by us and others.<sup>16-19 22</sup> Pelleted parasites were solubilised in 200 µl electrophoresis dissolving buffer<sup>29</sup> (125 mM TRIS-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% bromophenol blue) in the absence of β mercaptoethanol.<sup>16</sup> Samples (1 µl) were electrophoresed at 100 V in a minigel apparatus (BioRad Laboratories, Hercules, CA, USA) on 7% acrylamide copolymerised with 1.5 mg gelatin, as before.<sup>16-18 22</sup>

After SDS-PAGE, trichomonad proteinases were activated in proteinase activation buffer (PAB) (100 mM sodium acetate and 2.5% Triton-X 100, pH 5.5) prewarmed to 37°C containing varying concentrations of DTT. In this case, preparative slab gels were cut so that identical amounts of trichomonad protein were incubated separately in PAB containing specific amounts of DTT. Negative controls were incubated in PAB without any addition. PAB with hydrogen peroxide (10 µM) was also tested to determine the effect on proteinase activity.<sup>30 31</sup> Activation of proteinases was performed by placing gels on a shaker for 2 hours at 37°C with one change of buffer, and proteinase activity was seen after staining in Coomassie brilliant blue overnight followed by destaining for 2 hours in a solution of 7% acetic acid and 5% methanol.

### DENSITOMETRIC ANALYSIS OF SUBSTRATE GELS FOR DETERMINATION OF RELATIVE PROTEINASE ACTIVITY

All gels were digitalised with a flatbed scanner, and pixel intensity of each clear area representing a proteinase degradation band was quantified and graphed with the NIH Image software version 5.17. Percentage degradation for each band was calculated as follows:  $100 \div ((\text{no pixels scanned from gels activated at 1 mM DTT}) \times (\text{no pixels scanned from gels activated at } < 1 \text{ mM DTT}))$ .

## Results

### APPROXIMATION OF REDUCING LEVELS IN VWs

We did not know whether it would be possible to measure any reducing ability in VWs obtained and processed as described in Materials and methods. We further appreciated that any value of reducing level detected may not reflect that found at a specific site in the vagina parasitised by *T vaginalis*. None the less, the question regarding whether the vagina possessed an environment sufficient to activate trichomonad proteinases was important to attempt determination of in vivo reducing levels.

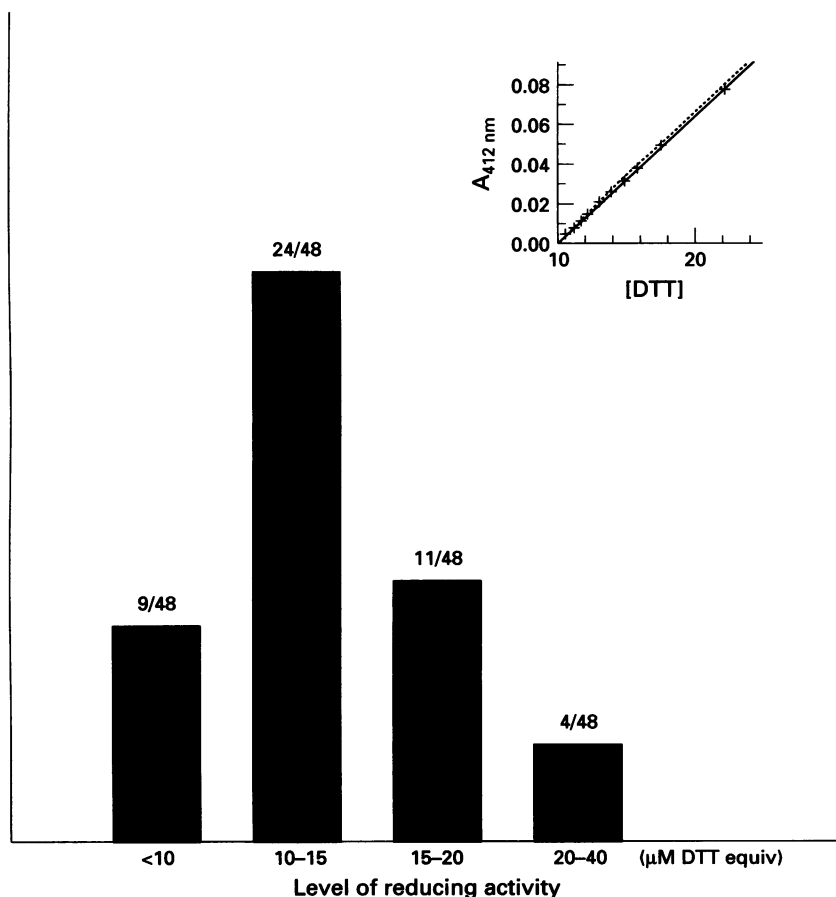


Figure 1 Distribution of reducing levels among the vaginal washes of patients with trichomonosis. On top of each bar are the numbers of samples positive for each range. A total of 48 vaginal washes was examined. The inset shows the standard curve using different concentrations of dithiothreitol (DTT), and a representative set of VWs analysed is represented by the plus signs. The protocol was as described in Materials and methods. Absorbance was monitored at 412 nm wavelength.

Figure 1 presents the results from the evaluation of 48 VWs and extrapolation of DTT equivalent levels from a standard curve using μM amounts of DTT (inset). The majority of the VWs (50%) had DTT reducing equivalents between 10 μM and 15 μM. Nine of 48 (18.7%) VWs assayed had ≤ 10 μM DTT equivalents. In contrast, 11 of 48 (23.0%) had

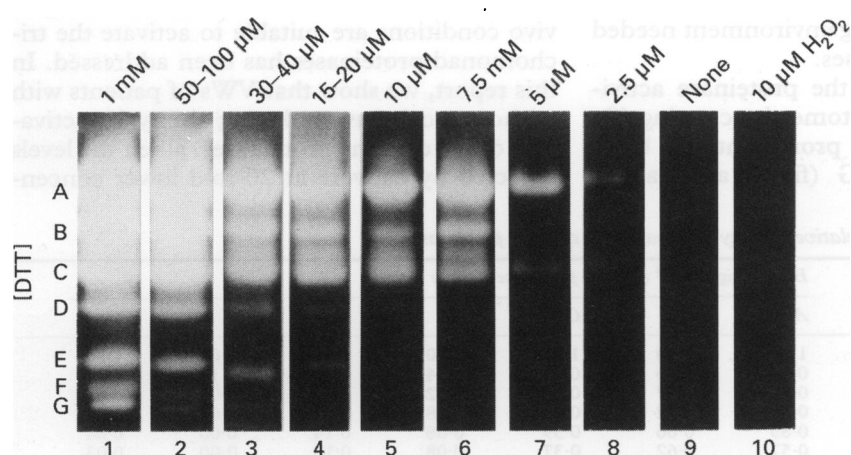


Figure 2 Representative cysteine proteinase patterns of *T vaginalis* by substrate gel electrophoresis with gelatin copolymerised with acrylamide using varying amounts of dithiothreitol (DTT) (lanes 1-8) in the activation buffer (Materials and methods). None refers to the control without any DTT (lane 9). Lane 10 shows the effect of addition of hydrogen peroxide ( $H_2O_2$ ) as described in the text. The letters on the left refer to proteinase band designations for comparative purposes as shown in figure 3 and in the table. Similar results were obtained with numerous isolates examined, including those listed in Materials and methods.

between 15 μM and 20 μM and four VWs (8.3%) had 20 μM to 40 μM DTT equivalents. This range (≤ 10 μM to 40 μM) was far lower than the 1 mM amount of DTT normally employed to examine the trichomonad cysteine proteinases in vitro.<sup>18 19 22</sup> None the less, these data show that VWs from patients do indeed possess reducing ability.

#### ARE VW REDUCING LEVELS SUFFICIENT TO ACTIVATE TRICHOMONAD CYSTEINE PROTEINASES?

We next wanted to determine whether cysteine proteinases were detected when activation was performed using concentrations of DTT within the range found in the VWs. In addition, because the manner in which the VWs were obtained and processed could yield lower values than those of undiluted vaginal secretions, we evaluated reducing levels from 10 μM up to 1 mM for comparative purposes.

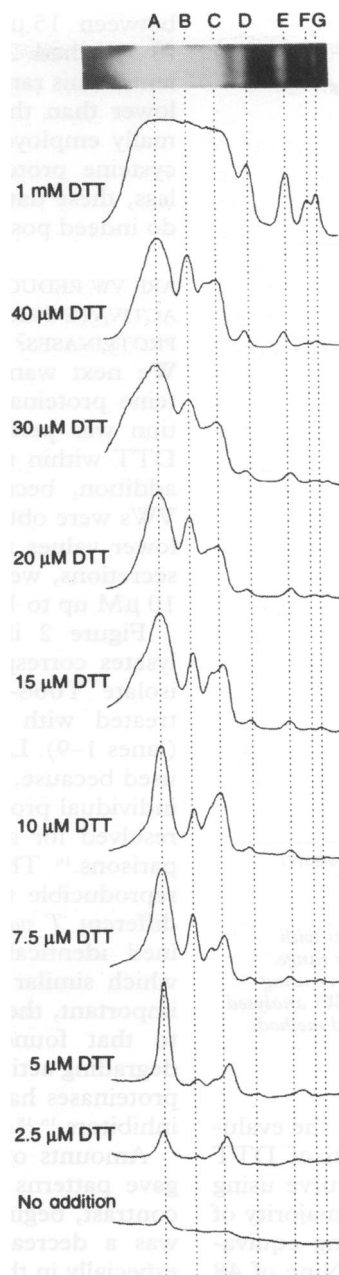
Figure 2 illustrates proteinase profiles of lysates corresponding to  $1 \times 10^5$  *T vaginalis* isolate T068-II organisms where gels were treated with decreasing amounts of DTT (lanes 1-9). Lysate of this parasite density was used because, by substrate gel electrophoresis, individual proteinase band activities were best resolved for accurate visualisation and comparisons.<sup>16</sup> This amount of lysate also gives reproducible total proteinase patterns among different *T vaginalis* isolates that were examined identically as presented here and for which similar results were obtained. Equally important, the number of parasites is relevant to that found in vivo.<sup>32</sup> Finally, that the degrading activities being analysed are cysteine proteinases has been confirmed using specific inhibitors.<sup>16-18 22</sup>

Amounts of DTT above 100 μM (lane 2) gave patterns similar to 1 mM (lane 1). By contrast, beginning at 100 μM (lane 2), there was a decrease in total proteinase activity, especially in the lower  $M_s$  (lane 1 versus lanes 3). Concentrations of DTT as low as 2.5 μM still gave detectable proteinase activity above background, as seen in lane 9 (greater than the negative control without addition of any reducing agent).

We had noticed that any basal level of proteinase activity observed in the negative controls could be readily neutralised by addition of 10 μM hydrogen peroxide in the activation buffer (lane 10). We, therefore, thought it would be important to determine whether hydrogen peroxide neutralised the activity of DTT pretreated cysteine proteinases. Hydrogen peroxide is known to reverse the effect of DTT,<sup>30 31</sup> and, as recently hypothesised, hydrogen peroxide producing *Lactobacillus* might therefore afford protection from *T vaginalis* infection.<sup>33-35</sup>

Identical experiments as above were performed in substrate gels that were activated in 40 μM DTT, the minimum concentration found in VWs that gave good activation of the trichomonad cysteine proteinases (fig 2), except that activation buffer incorporated varying amounts of  $H_2O_2$ . At 200 μM  $H_2O_2$ , no activation of proteinases was detectable,

Figure 3 Densitometric scanning of the proteinase bands obtained after activation at the designated dithiothreitol (DTT) concentrations. The surface under each peak was analysed as described in Materials and methods for quantitative presentation in the table. The dotted line was used to align each peak.



showing that hydrogen peroxide neutralised the action of the reducing environment needed for the cysteine proteinases.

To better quantitate the proteinase activities, we performed densitometric scanning (fig 3) and evaluated each prominent gel band region labelled A to G (fig 3 and table).

Proteinase bands F and G were greatly decreased by 100  $\mu$ M DTT (table) and were reduced in activity by 90% at 50  $\mu$ M DTT. Prominent substrate degradation by proteinase bands D and E were also strongly affected by a decline in DTT concentration; a  $\sim$ 50% reduction in proteolysis occurred by 100  $\mu$ M DTT, and  $\sim$ 80% activity was diminished by 50  $\mu$ M DTT. Band C was decreased 50% in activity by 50  $\mu$ M DTT, while band regions A and B approached a 50% reduction in activity by 20  $\mu$ M DTT. Therefore, when evaluating the level of DTT at which a 50% reduction in activity is detected, differences are apparent among the proteinase band regions.

## Discussion

The interrelation between the host and *Trichomonas vaginalis* is exceedingly complex, and some of the many factors that contribute to such complexity include the multiple cysteine proteinases that potentially contribute to host cytopathology. We have previously hypothesised that the in vivo synthesis of the proteinases must somehow be under the control of environmental cues so as to modulate the number and amount of proteinases needed at any particular moment of infection. In this scenario only that quantity of proteinases necessary for parasite survival and which provoke minimal host damage would be both present and active. Recent evidence shows the induction by iron of proteinases with specific functions, such as degradation of complement C3 to avoid lysis through the alternative pathway.<sup>13</sup> In addition to regulatory paths that control the synthesis of the trichomonad proteinases, we now present the possibility that host reducing levels may also regulate the activity of the proteinases. This would provide another mechanism of control over the many proteinases produced by *T vaginalis*.

Of importance was the finding that VWs indeed possess reducing ability. To our knowledge, this may be the first time that the question regarding whether, during infection, in vivo conditions are suitable to activate the trichomonad proteinases has been addressed. In this report, we show that VWs of patients with trichomonosis have reducing ability for activation of the cysteine proteinases, albeit the levels detected by us were at 20-fold lower concen-

The effect of reducing level on relative activity of *T vaginalis* cysteine proteinases

In vivo range	DTT ( $\mu$ M)	Band designation* (relative proteinase activity †):						
		A	B	C	D	E	F	G
—	1000.0	1.00	1.00	1.00	1.00	1.00	1.00	1.00
—	100.0	0.84	0.96	0.87	0.54	0.44	0.19	0.26
—‡	50.0	0.80	0.97	0.52	0.22	0.17	0.09	0.09
+‡	40.0	0.65	0.72	0.52	0.18	0.18	0.04	0.09
+	30.0	0.85	0.66	0.39	0.08	0.14	0.00	0.04
+	20.0	0.57	0.62	0.37	0.08	0.10	0.00	0.03
+	15.0	0.67	0.46	0.46	0.08	0.13	0.00	0.00
+	10.0	0.45	0.27	0.45	0.00	0.00	0.00	0.00
+	7.5	0.51	0.39	0.30	0.00	0.00	0.00	0.00
+	5.0	0.25	0.06	0.17	0.00	0.00	0.00	0.00
+	2.5	0.07	0.03	0.13	0.00	0.00	0.00	0.00

\*Relative activity was calculated as described in Materials and methods.

†Band designations were from the individual areas of proteinase activity as presented in figures 2 and 3.

‡Minus and plus signs refer to the levels of reducing ability in DTT equivalents as determined from the evaluations of vaginal washes as shown in figure 1 and described in Materials and methods.

trations than those used in vitro.<sup>16-19 22</sup> It is conceivable that the lower amounts of reducing levels represent a dilution resulting from obtaining and/or processing the VW. Regardless, it is clear that lower amounts of reducing ability produce qualitatively and quantitatively decreased activity of parasite proteinases when compared with 1 mM DTT. Finally, it might have been predicted that all the proteinase activities would decrease equally at a particular reducing level. That this was not the case reaffirms the distinctness among the numerous cysteine proteinases, as suggested earlier.<sup>17</sup>

It is essential that we begin to understand the host factors and parameters within the site of infection that contribute to individual susceptibility and resistance to infection. The hypothesis that women with higher vaginal reducing ability may be more susceptible to parasitism and cytopathology seems reasonable, especially given the relation between qualitative and quantitative proteinase activities and differential reducing levels. This idea is plausible since proteinase activity is necessary for survival of *T vaginalis* through nutrient acquisition,<sup>21</sup> cytoadherence,<sup>12</sup> and immune evasion.<sup>13 16</sup>

If the hypothesis presented above is correct, then relative resistance by men to infection by *T vaginalis* may be related to the presence of oxidising reagents that prevent activation of the trichomonad cysteine proteinases essential for survival. In fact, reactive oxygen species produced by human spermatozoa have been described,<sup>36 37</sup> and this oxidative environment would not allow for host parasitism and establishment of a non-self limiting infection.

We showed the action of hydrogen peroxide in neutralising the reducing ability of DTT, which is a prerequisite for activation of the cysteine proteinases. Our results reinforce the notion that hydrogen peroxide producing *Lactobacillus* may confer some measure of defence against *T vaginalis*. This is only possible, however, if sufficient amounts of hydrogen peroxide are produced at and bathe the site of infection. The idea to test for neutralisation of trichomonad proteinases resulted from the known reversible effect by hydrogen peroxide on systems requiring reducing environments.<sup>30 31 38</sup> Furthermore, the idea may have merit because of the reports that the host may be protected from other sexually transmitted diseases by hydrogen peroxide producing *Lactobacillus*.<sup>33-35</sup> Although reversible, demonstration of hydrogen peroxide neutralisation of the activation power of DTT on trichomonad proteinases suggests that an approach for intervention of *T vaginalis* infection and cytopathology may result from generation of irreversible proteinase inhibitors or, alternatively, upon inhibition of the ability of the parasite to alter the normal protective flora of patients.

Based on the hypothesis proposed above, numerous questions require attention. (1) Does the level of vaginal reducing ability vary during menses, and, if so, are certain times during the cycle more favourable to proteinase

activation, which then promotes host parasitism? (2) Does the relative reducing level in the vagina vary among different ethnic groups, thus explaining higher rates of trichomonosis among African American women?<sup>6</sup> (3) Does behaviour that predisposes women to *T vaginalis* infection, such as smoking,<sup>6</sup> alter the vaginal reducing environment? (4) Is there a relation between the level of reducing ability, parasite growth and multiplication in vivo, and symptomatology? (5) Does the relative absence of symptomatology and the self limiting nature of infection among males correlate with absence of reducing ability at those sites? Our results provide a foundation from which testable hypotheses can be formulated and that may, ultimately, help us understand those important host factors pertinent to infection by this sexually transmitted agent.

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